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Transcriptional Analysis and Preliminary Characterization of ORF Bm42 from *Bombyx mori* Nucleopolyhedrovirus

Asha Acharya and Karumathil P. Gopinathan[1]

Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560012, India

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Clusters of closely spaced genes are frequently present in baculovirus genomes. We report here the detailed transcriptional mapping of one such gene cluster from *Bombyx mori* nucleopolyhedrovirus (BmNPV) comprising five ORFs, viz., Bm42, Lef-10, VP1054, Bm44, and Bm45, which are closely spaced and transcribed in the same orientation. This region is homologous to ORFs 53, 53a, 54, 55, and 56, respectively, on the AcMNPV genome. Multiple, overlapping sets of polycistronic transcripts from this region, that initiate independently but terminate at a common 3′ end, were detected following BmNPV infection. Expression of these genes followed a temporal pattern where the shorter transcripts initiating downstream appeared early, and with the progress of infection the longer transcripts initiated from upstream. The first ORF in this cluster, Bm42, is conserved in almost all baculoviruses, but has not been characterized so far. The protein was localized in the cytosol, predominantly near the nucleus-cytoplasm boundary in BmNPV-infected BmN cells. Bm42 was present in the budded virions and was not associated with occluded virus particles or the polyhedral matrix.

Key Words: baculovirus; BmnPV; immunolocalization; polycistronic message; promoter occlusion; transcriptional regulation.

INTRODUCTION

The *Baculoviridae* family of occluded insect viruses harbor covalently closed double-stranded circular genomes and stabilize their virions outside the insect host in a crystalline protein matrix composed predominantly of the viral very late occlusion body protein, polyhedrin (Blissard and Rohrmann, 1990; King and Possee, 1992; Hayakawa *et al.,* 2000). *Autographa californica* multinucleocapsid nucleopolyhedrovirus (AcMNPV) is the most extensively studied member of this family (Miller, 1992; Ayres *et al.,* 1994; Possee, 1997). *Bombyx mori* nucleopolyhedrovirus (BmNPV) has emerged as a parallel system to AcMNPV for the high-level expression of recombinant genes in insect caterpillars (Maeda, 1989a,b; Palhan *et al.,* 1995; Sumathy *et al.,* 1996; Sehgal and Gopinathan, 1998; Acharya and Gopinathan, 2001). Gene expression in baculoviruses follows a temporal cascade (Friesen and Miller, 1986) and the onset of viral replicative cycle is preceded by the transcription of viral early genes by the host-cell RNA polymerase. Transcription of late and very late genes follows viral DNA replication and is mediated by an α-amanitin- and tagetitoxin-resistant virally encoded RNA polymerase, initiating from a tetranucleotide "TAAG" motif (Fuchs *et al.,* 1983; Possee and Howard, 1987; Yang *et al.,* 1991).

Changes in the pattern of viral protein synthesis during baculovirus infection closely correlates with the appearance of specific viral RNAs, supporting the contention that regulation of gene expression is primarily mediated at the level of transcription. Several regions in the AcMNPV genome that encode nested sets of overlapping transcripts with common 5′-initiation or coterminal 3′-polyadenylation sites have been identified (Lubbert and Doerfler, 1984a,b; Friesen and Miller, 1985; Rankin *et al.,* 1986; Oellig *et al.,* 1987). Transcription of RNAs with 3′-coterminal nests follows an unusual pattern involving early transcription of the smallest RNAs followed later by transcription of successively longer RNAs mapping farther upstream. One such nest includes the *Hin*dIII B_2 and the *Hin*dIII K units of AcMNPV where the length of transcripts from the respective genomic segments increases with time and concomitantly the relative abundance of the shorter transcripts decreases (Friesen and Miller, 1985, 1986). The mechanism of sequential recognition of distal transcription initiation sites (i.e., the initiation sites located farther upstream at late times), which blocks access of the RNA polymerase to downstream promoters by steric hindrance or by localized distortion of the DNA structure, has been described as "promoter occlusion" (Hausler and Sommerville, 1979) but so far there is no direct evidence for its operation in baculoviruses. Another region encompassing the *Eco*RI J and N fragments of the AcMNPV genome (map units 81.2 to 85.0) has been reported to give rise to at least nine classes of RNAs ranging in size from 1.3 to 4.6 kb, with common 3′-termini (Oellig *et al.,* 1987). Once again, the larger transcripts were present only at late times. Promoter

[1] To whom correspondence and reprint requests should be addressed. Fax: 91(80) 360 2697. E-mail: kpg@mcbl.iisc.ernet.in.

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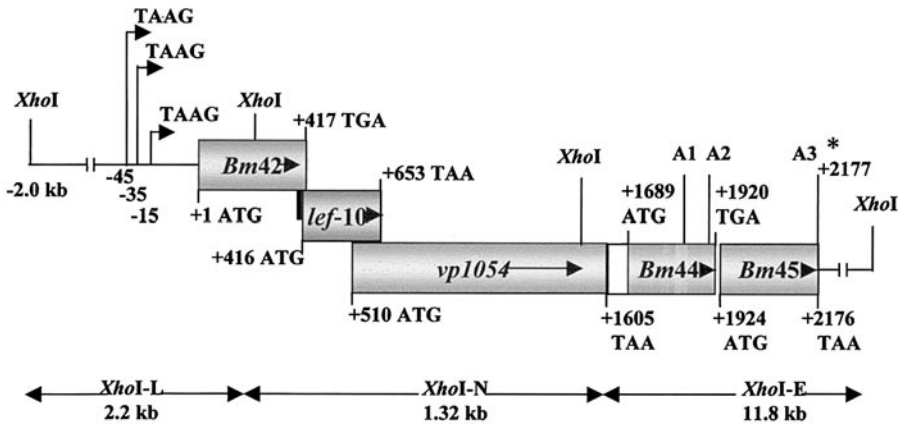


FIG. 1. Genomic organization of the BmNPV *Bm*42 region. The arrangement of genes on the BmNPV genome between 30.5 and 32.4 m.u. (nt 37,756 to 53,104 on BmNPV genome) is shown. The +1 ATG of Bm42 corresponds to nt 39,790 on the BmNPV genome. Predicted ORFs (Bm42, Lef-10, VP1054, Bm44, and Bm45) are clustered and are in the same orientation as marked (→). The location of *Xho*I-restriction sites depicting the cloned library fragments "L" and "N" and beyond (fragment "E") are indicated. The translational start and termination codons of all the ORFs in this cluster are marked and numbered in relation to the +1ATG of Bm42, at the beginning and end of each ORFs. The overlaps between ORFs Bm42 and Lef-10, and Lef-10 and VP1054, are also shown. The Bm42 region corresponds to ORFs Ac53 to Ac56 on the AcMNPV genome. The canonical late transcription start motifs "TAAG" (located at −15, −35, and −45 with respect to the +1 ATG of the *Bm*42) as well as the polyadenylation signal sequences "AATAAA" (marked A1, A2, and A3*) are indicated.

occlusion has been documented in various nonviral and viral systems, including the human α-globin gene (Proudfoot, 1986), rRNA genes (Bateman and Paule, 1988; Moss *et al.,* 1992), adenovirus polypeptide IX mRNA (Vales and Darnell, 1989), and the HIV-1 promoter (Gregor *et al.,* 1998). In contrast, overlapping transcripts with common 5′ ends have been described for the two major late genes, *polh* and *p10* from AcMNPV (Friesen and Miller, 1985; Rankin *et al.,* 1986), which result from transcriptional readthrough of several polyadenylation signals located 3′ to the smaller transcript. These transcripts have been speculated to indirectly down-regulate the expression of downstream genes by an antisense mechanism. Such transcription nests in these viruses are believed to have evolved as a consequence of the strong pressure to economize on the genome size.

Analysis of the BmNPV genome sequence (Gomi *et al.,* 1999) revealed several regions consisting of tandemly located overlapping or nonoverlapping ORFs with short intergenic regions and in the same orientation. We report here the transcriptional analysis of one such locus, viz., the BmNPV *Bm*42 region. This locus, in addition to ORF Bm42, comprises *lef*-10 (ORF Bm42a), *vp*1054, ORF Bm44, and ORF Bm45, all of which are located in the same orientation and have overlapping reading frames or are separated by very short intergenic region. The AcMNPV counterpart for this region includes ORFs 53, 53a, 54, 55, and 56, respectively. Our studies reveal the presence of multiple sets of overlapping transcripts, which terminate at a common 3′ end. We also document the localization of Bm42 protein in the cytoplasm of BmNPV-infected BmN cells and as a component of the budded virus.

RESULTS

Sequence analysis of the *Bm*42 region of BmNPV

In BmNPV, the *Bm*42 region harbors five ORFs, encompassing ORFs Bm42, Lef-10 (Bm42a) VP1054, ORF Bm44, and Bm45 (Gomi *et al.,* 1999) as presented schematically in Fig. 1. Bm42, the first gene in this cluster [nucleotides (nt) 39,790 to 40,207 on the BmNPV genome], harbors three of the canonical late transcription start motif TAAG in its immediate upstream and potentially codes for a 139-aa-long polypeptide. The functional role of this gene, highly conserved in most baculovirus sequenced so far, is not known. The +1 ATG start codon of Lef-10, present immediately downstream and in the same orientation (located between nt 40,206 and 40,440 on the BmNPV genome), overlaps with the translation termination of Bm42 by 2 nt. The coding sequences of Lef-10 ORF and the immediate downstream gene *vp*1054 (nt 40,300 to 41,395 on the BmNPV genome) overlap by 140 nt but read in a different frame. The +1 ATG of *vp*1054 is located at +94 nt from the translational start of Lef-10. Both *lef*-10 and *vp*1054 lack the canonical early (CAGT)- or late (TAAG)-transcription start site (tss) motifs in their immediate upstream. Three potential polyadenylation signal sequences (AATAAA) are present in the downstream region; two of these, separated from each other by 52 nt, are located within the coding region of *Bm*44, and the third one overlaps with the translational termination codon of *Bm*45. This Bm42 region of BmNPV is homologous to the ORF53–ORF55 region of the AcMNPV genome. Of the five ORFs in this region, only Bm42 and VP1054 are conserved in most baculoviruses (nucleopolyhedroviruses and granulosis viruses) sequenced so

TABLE 1

Oligonucleotides Used in the Study

Primer	Sequence	Purpose
P1	5′ cgggaattcggatccatgttctgcaccgtttg 3′ (39790–39806)*	*Bm*42 +1 ATG forward primer for PCR to generate protein expression clones, and RT-PCR
P2	5′ gggcattgccactatgcc 3′ (39927–39910)	tss mapping of *Bm*42 by primer extension
P3	5′ cgctagaggaatgcaacg 3′ (40052–40069)	*lef*-10 forward primer for RT-PCR
P4	5′ gcgtcgactgattgcattttaaaaaattcc 3′ (40206–40184)*	*Bm*42 reverse primer lacking translational stop
P5	5′ ccggaattcatgacgaacgtatgg 3′ (40206–40220)*	*lef*-10 forward primer for RT-PCR
P6	5′ cttgggcggccgcatcgg 3′ (40383–40366)	Primer extension of *lef*-10 and *vp*1054
P7	5′ ggtctgcacgcacacaacgtg 3′ (41374–41394)	Nested forward primer 1 for 3′ RACE
P8	5′ cgggtgccagtgcaaaattgag 3′ (41576–41597)	Nested forward primer 2 for 3′ RACE
P9	5′ atctcgagtgtgttcgcg 3′ (40005–39988)	Reverse primer at *Xho*I site in *Bm*42
P10	5′ gcgtcgacgtggacgcgttactttg 3′ (40438–40422)*	Reverse primer at *lef*-10 C-terminal end for RT-PCR
P11	5′ tatacatatgttctgcaccgtttg 3′ (39790–39806)*	Forward primer for expression of *Bm*42 from the native +1 ATG of the gene

Note. Oligonucleotides used in various PCR amplifications, primer extensions, and 3′ RACE are listed. The numbers in parentheses indicate the coordinates on BmNPV genomic DNA. *denotes the presence of linker sequences.

far. In AcMNPV, *vp*1054 has been shown to encode a viral structural protein that is involved in nucleocapsid assembly (Olszewski and Miller, 1997). The gene *lef*-10 is absent in the granulosis virus PxGV (Hashimoto *et al.,* 2000). Functions of the other two downstream ORFs, Bm44 and Bm45 (located at nt 41,479 to 41,710 and nt 41,714 to 41,966, respectively, on the BmNPV genome), are not known.

Transcriptional mapping of the BmNPV *Bm*42 region

To check if the three canonical TAAG late-transcription start motifs in the upstream region of *Bm*42 were utilized in initiation of transcription, primer extension reaction was carried out using the primer P2 (all the primer sequences and their locations on BmNPV genomic DNA are listed in Table 1). Transcripts emerging from this region comprised two doublets (~152 and 172 nt) and a longer signal at 182 nt (Fig. 2a). The shorter doublet corresponded to the "T" and first "A" of the TAAG sequence at −15 nt from the +1 ATG of ORF Bm42. The longer doublet matched with an identical late motif present at −35 nt from Bm42 +1 ATG codon. The largest of the extended products corresponded to the T of the TAAG at −45 nt position. Thus all the three consensus late transcription start motifs "TAAG" located upstream of the ORF appeared to be utilized for initiation.

To determine the transcription start points of the downstream genes, *lef*-10 and *vp*1054, primer extension was carried out with primer P6. Multiple products of ~180, 340, and 600 nt detected were mapped precisely using the sequencing ladder generated from the same region using the same primer. The shortest of the extended product mapped to the "G" and "C" of a TGCA motif located immediately upstream of the Lef-10 +1 ATG (Fig. 2b). The 340-nt signal corresponded to the first "G" of a GCGC sequence at −163 nt from the +1 ATG of *lef*-10 (Fig. 2c). Presence of this transcript was also confirmed by an RNase protection assay performed in the presence of the DNA replication inhibitor, aphidicolin, using the antisense RNA-strand corresponding to the C-terminal 208 bp of the ORF Bm42 (Fig. 2d). A 163-nt protected fragment was detected at 12 and 18 h.p.i., which corresponded to the transcript arising from the transcription start site (GCGC) identified in the primer extension analysis. The ~600-nt-long primer extended product (size roughly estimated from the labeled molecular size markers) corresponded to the transcripts encompassing *Bm*42 (also detected in primer extensions with primer P2, data not shown). The extended products detected from primer P6 suggested that all of them corresponded to polycistronic messages. The results from 5′-end analysis of the various ORFs are summarized in Fig. 2e along with the genomic sequence of the *Bm*42 region and are summarized schematically in Fig. 3 (top).

Further, to determine whether the transcripts identified by primer extension were early or late, RT-PCR was

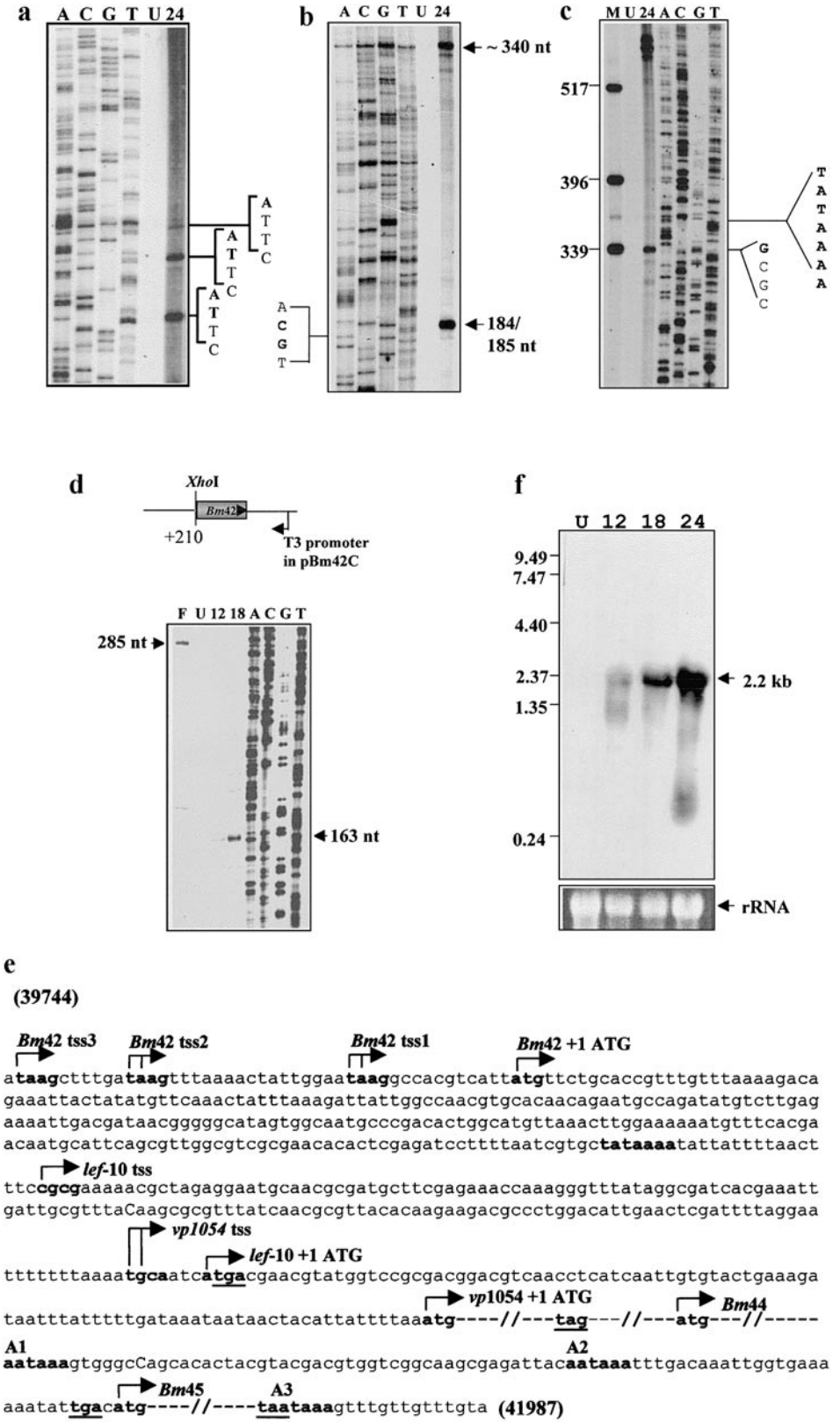


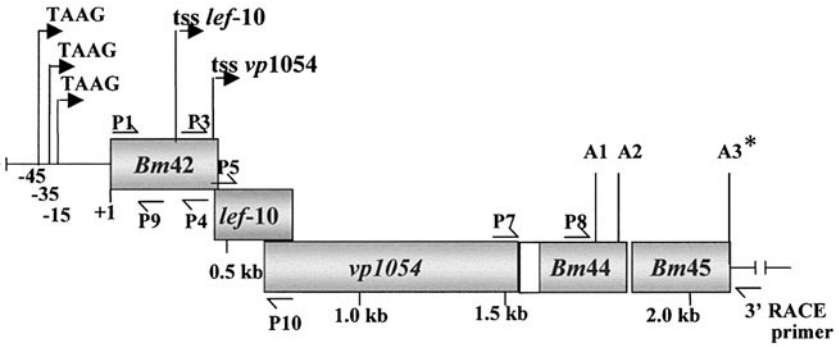
FIG. 2. Transcriptional analysis of the *Bm*42 region. (a) Primer extension. 5′-end mapping of ORF Bm42 was carried out using primer P2 (for primer sequences, see Table 1) located at +138 nt from the +1 ATG of Bm42. Total RNA (20 μg) isolated from uninfected (lane U) or BmNPV-infected BmN cells (m.o.i. of 10) at 24 h.p.i. (lane marked 24) was hybridized to primer P2. Reverse transcription was performed using Superscript reverse transcriptase in the presence of [α-^{32}P]dATP and the products were analyzed on a 7 M urea–6% polyacrylamide gel, followed by autoradiography. The transcription start sites (tss) were mapped based on the sequencing ladder generated for *Bm*42 (clone pBmXL harboring the 2.2-kb *Xho*I "L" fragment)

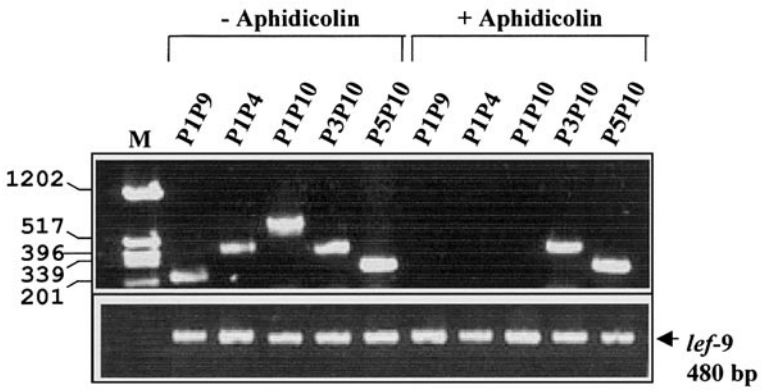
performed using different sets of primer combinations with the RNA isolated from BmNPV-infected cells in the presence and absence of the DNA replication inhibitor aphidicolin (Fig. 3). The appropriate locations of the primers are indicated schematically in the top panel and the expected sizes of the amplified products using the various primer combinations as well as the specificity of detection of the transcripts are also presented in a tabular form. The time of appearance of the transcripts, whether early (insensitive to aphidicolin) or late (sensitive to aphidicolin), is also provided in this table. The observed and predicted sizes of the transcripts agreed well (see the PCR amplification pattern). The transcripts initiating upstream of *Bm*42 were sensitive to aphidicolin confirming late transcription. On the contrary, the transcripts corresponding to *lef*-10 and *vp*1054 were equally intense in the presence or absence of aphidicolin, showing that they were indeed early transcripts.

To determine the total size of the *Bm*42 transcript, Northern blot was performed using the total RNA isolated from the BmNPV-infected BmN cells at 12, 18, and 24 h.p.i. (Fig. 2f). Using a probe specific for *Bm*42 (210-bp PCR-amplified product of P1 and P9 from BmNPV genomic DNA corresponding to the 5′ end of the gene and nonoverlapping with the *lef*-10 or *vp*1054 transcripts), a 2.2-kb signal was detected. This signal was weak at 12 h.p.i. but increased significantly by 24 h.p.i. The observed size of this signal supported the notion of a polycistronic message and suggested that the two downstream ORFs (Bm44 and Bm45) were also part of this polycistronic transcript.

To confirm this and to investigate whether the three polyadenylation signals identified based on sequence analysis in this region were utilized for transcription termination, 3′-RACE reaction was performed with the total RNA isolated from BmNPV-infected BmN cells. To ensure that the amplification covered the transcript corresponding to *Bm*42, the first round of PCR was performed using primer P1 (carrying the +1 ATG of ORF Bm42) and 3′-RACE reverse primer (3′-RACE RP; see schematic presentation in Fig. 3 for primer locations and their actual positions on the BmNPV genome in Table 1). The amplified product was reamplified first using a nested primer P7 and 3′-RACE RP and then with a second nested primer P8 in combination with the 3′-RACE RP, which generated a single ~450-bp fragment. The sequence analysis of this fragment showed that polyadenylation (27 A residues) took place at the last residue (nt 41,987), marked in Fig. 2e. Clearly, the distal-most AATAAA sequence, located ~600 nt downstream of the *vp*1054 translational termination codon, was the signal used for polyadenylation (marked A3 in Fig. 2e and diagram in Fig. 3). The other two potential signal sequences (marked A1 and A2 in Fig. 2e) were present within the PCR-amplified fragment. No shorter amplification products corresponding to these polyadenylation sequences (A1 and A2), if used for transcription termination, were detected.

using the same primer (lanes marked A, C, G, and T). The tss are indicated and the corresponding sequences are provided next to the sequencing ladder. The start sites are marked in bold and the sequences generated (marked on the right) correspond to that of the complementary strand. (b) and (c) tss mapping of BmNPV *vp*1054 and *lef*-10. Primer extension was performed with primer P6 located 176 nt downstream of the +1 ATG of *lef*-10. The extended product of ~180 nt was fine mapped using a sequencing ladder generated from the same region (pBmXN, harboring the 1.32-kb *Xho*I "N" genomic fragment of BmNPV and primer P6; lanes marked A, C, G, and T). The tss mapped to a doublet at nucleotides 184/185 is indicated by an arrow and the corresponding sequences are provided next to the sequencing ladder (b). The primer extended product of 340 nt was fine mapped based on the same sequencing ladder by running the gel longer. The tss and the TATA box-like region are indicated and the corresponding sequences are provided. The start site has been marked in bold in both cases. (d) Transcription start site mapping of *lef*-10. The tss of *lef*-10 was confirmed by RNase protection analysis using the labeled RNA probe, antisense to the region encoding the C-terminal region Bm42. The generation of antisense RNA is shown schematically on the top of the panel. Plasmid construct pBm42C harboring 208 bp from the *Xho*I-restriction site to the 3′ end of *Bm*42 was linearized with the *Xho*I and an antisense riboprobe of 285 nt was generated *in vitro* using T3 RNA polymerase. Total RNA (20 μg) from uninfected (lane U) and BmNPV-infected BmN cells (treated with 50 μg aphidicolin/ml), at 12 and 18 h.p.i. (lanes marked 12 and 18) was hybridized with the antisense riboprobe at 50°C in 50% formamide for 12 h followed by digestion with RNase A (2U) and RNase T1 (1U). The samples were electrophoresed on 7 M urea–6% acrylamide gel and the protected fragments were detected by autoradiography (indicated by arrow). The fragment was sized using a sequencing ladder generated from an unrelated known sequence. (e) Sequence of the *Bm*42 region of BmNPV. Partial sequence of the BmNPV genome starting from nt 39,744 to 41,987 is shown. The transcription start sites from *Bm*42 (marked tss1, tss2, and tss3), *lef*-10, and *vp*1054 are indicated. The translational start sites (+1ATG) for all the five ORFs in this region are marked in bold. The translational termination site (TGA) of Bm42 overlapping with the start codon (+1ATG) of Lef-10 as well the translation termination codons of VP1054, Bm44, and Bm45 (TAG, TGA, and TAA, respectively) are marked in bold and underlined. The putative TATAA box sequence upstream of the *lef*-10 tss is also shown in bold. The potential polyadenylation signals A1, A2, and A3 are marked. A1 and A2 are located within the *Bm*44 coding region, whereas A3 overlaps by 2 nt at the translational termination codon of Bm45. The poly(A) stretch (27 residues) is added at nt 41,987. (f) Northern analysis of *Bm*42 transcript. Total RNA (30 μg) isolated from uninfected (lane U) and BmNPV-infected BmN cells (m.o.i. 10) at 12, 18, and 24 h.p.i. (lanes marked 12, 18, and 24) was resolved on a 1.2% MOPS-formaldehyde agarose gel and transferred on to nylon membrane. Hybridization was carried out for 16 h at 42°C in the presence of 50% formamide using a probe specific to the *Bm*42 5′-terminal region, generated by PCR amplification with primers P1 and P9 (nt 39,791 to 39,995 on the BmNPV genome) that was nonoverlapping with *lef*-10 5′-UTR. The probe was radiolabeled by random priming and after hybridization the blot was washed at 65°C for 1 h in the presence of 0.5× SSC and 0.1% SDS and autoradiographed. The signal corresponding to *Bm*42 transcript is marked by an arrow. To indicate the balanced amounts of RNA loaded, an ethidium bromide staining of the same gel showing the 28S rRNA levels is shown below.





Primer combination	Expected size (bp.)	Specificity for transcript	Appearance of transcript
P1-P9	215	*Bm*42	Late
P1-P4	416	*Bm*42 and *lef*-10	Late
P1-P10	648	*Bm*42, *lef*-10 and *vp*1054	Late
P3-P10	386	*lef*-10 and *vp*1054	Early
P5-P10	236	*vp*1054	Early

FIG. 3. Detection of early and late transcripts in *Bm*42 region. Total RNA isolated from BmNPV-infected BmN cells (m.o.i. 10) treated or untreated with aphidicolin was reverse transcribed in the presence of oligo(dT) anchor primer and Superscript reverse transcriptase at 42°C for 1 h. The first-strand cDNA was PCR amplified using different combinations of primers (e.g., P1 and P9 or P1 and P4 as marked over each lane). The primer locations are schematically shown on the top panel. (For primer sequences, see Table 1.) The approximate genomic sizes are marked with reference to the +1 ATG of *Bm*42. The expected sizes of amplified products with various primer combinations and their specificity in the detection of the transcript as well as the actual appearance of the transcripts are presented in tabular form. Lane M, size marker pTZ DNA digested with *Hin*fI. As a control, the amplification of an early transcript, *lef*-9 (A. Acharya and K. P. Gopinathan, unpublished results) was carried out on the same cDNA using cognate primers and is shown as the lower panel. The locations of primers P7 and P8, and the 3′ RACE primer used in 3′ end mapping of the transcripts, are also indicated schematically in the cartoon.

Synthesis and immunolocalization of Bm42 in BmNPV-infected BmN cells and budded virus

The temporal profile of Bm42 synthesis following BmNPV infection was analyzed by Western blotting. Antibodies to Bm42 were raised against the His-tagged, thioredoxin-fusion protein, overexpressed in *Escherichia coli* (Fig. 4a). The bacterially expressed, His-tagged, thioredoxin fusion protein gave a single sharp band of 34 kDa, which was the predicted size. An ~18-kDa doublet band, corresponding to Bm42, was seen in BmN cells, faintly detectable at 12 h.p.i., which increased in intensity with the progress of infection (Fig. 4b). The presence of multiple bands could be due to posttranslational modifications or partial proteolysis.

Immunolocalization of Bm42 in BmN cells following BmNPV infection using the polyclonal antibodies detected the protein almost exclusively in the cytoplasm of BmNPV-infected cells (red) and the large nuclear region (blue) was nearly free of the expressed protein (Fig. 5, top row). However, Bm42 appeared to be most concentrated near the nucleus cytoplasmic boundary, as seen from the superimposition of the staining patterns (Fig. 5). As controls for staining, Lamin B, an inner nuclear membrane protein and human growth hormone (hGH), a cytosolic protein (expressed through recombinant BmNPV infection) (Fig. 5, middle rows, as marked), as well as uninfected BmN cells stained with Bm42 antibodies (Fig. 5, bottom row), were included. The marking of the inner nuclear membrane by lamin staining can be clearly distinguished from the intense outer nuclear membrane-cytoplasmic boundary staining by Bm42 antibodies (yel-

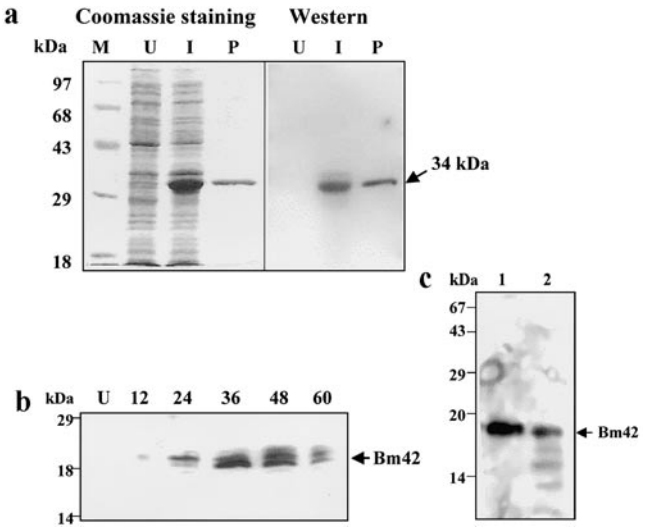


FIG. 4. Synthesis of Bm42 in BmNPV-infected BmN cells and its association with the budded virus. (a) Overexpression of Bm42 in bacteria. The Bm42 ORF lacking the translational stop codon was amplified from BmNPV genomic DNA using primers P1 and P4 and cloned into pET32a as a 417-bp *Bam*HI-*Sal*I fragment. Bacterial cultures were induced with 25 μM IPTG at 22°C for 8 h (lane I, compare with the uninduced lane U) and the induced protein (predicted size, 34 kDa inclusive of the tag) was purified on Ni-NTA agarose matrix (lane P). Polyclonal antiserum to the purified protein was raised in rabbit by standard protocol and the crude uninduced and induced (lane U′ and I′) as well as purified (lane P′) samples were Western blotted (shown on right). Bm42 protein without the purification tag was also expressed in bacteria (see c, Lane 1). (b) Temporal profile of Bm42 synthesis in BmNPV-infected BmN cells. Total cell lysate from 1×10^5 BmN cells uninfected (Lane U) or BmNPV-infected (Lanes 12, 24, 36, 48, and 60 h.p.i.) was resolved by electrophoresis on 0.1% SDS–15% polyacrylamide gel. The resolved proteins were transferred on to a PVDF membrane. The blot was blocked by treatment with 0.3% gelatin overnight and probed using anti-Bm42 rabbit antiserum followed by binding to goat anti-rabbit IgG conjugate to HRP. The signals due to HRP reaction were visualized using ECL+Plus Western blot detection kit (Amersham Pharmacia Biotech). (c) Detection of Bm42 on budded virus. Purified preparation of budded viruses were suspended in Laemmli buffer and loaded on a 0.8% SDS–15% acrylamide gel, followed by Western blotting with anti-Bm42 antibodies. The Western blot was developed using the ECL+Plus kit (Amersham Pharmacia Biotech). Lane 1: bacterially expressed Bm42 (without purification tags), solubilized from inclusion bodies by treatment with guanidinium hydrochloride; Lane 2: budded virus preparation of BmNPV.

low color in the overlay) or the exclusive cytoplasmic location of hGH.

Further, the association of Bm42 protein with the budded or occluded virions was examined. Western blotting of the proteins from the purified budded virus preparation, using anti-Bm42 antibodies, detected a protein band corresponding to that seen with overexpressed and purified protein (Fig. 4c, Lane 2). To confirm the specificity of Bm42 antibodies, we had also expressed the protein in bacteria, without His tag and thioredoxin fusion (see Fig. 4c, Lane 1). A single 18-kDa band corresponding to Bm42 is clearly seen in immunoblots. The presence of partially degraded protein detected from the virus (Lane 2) could have arisen during the virus purification steps. Under these conditions, however, the presence of Bm42 was not detected with the polyhedra-derived virus or the polyhedral matrix in Western blots. Bm42 is thus associated with the budded virion structure.

DISCUSSION

Clusters of tandemly arranged genes in the same orientation are a common occurrence in baculovirus genomes. A characteristic transcription pattern is followed in such clusters, which involve independent initiation of transcription for each of the genes in the cluster, while the termination occurs mostly at a common 3′ end. The transcription pattern of the BmNPV *Bm*42 region, which harbored one such cluster of closely spaced five ORFs oriented in the same polarity in tandem, has been analyzed here in detail. The first two ORFs of this cluster, Bm42 and Lef-10, overlapped by 4 nt, while the overlap between Lef-10 and the immediate downstream gene encoding the nucleocapsid protein VP1054 was 140 nt. The latter two, however, were in different reading frames. The two farthest downstream ORFs in this cluster Bm44 and Bm45 were separated by just 4 nt.

Primer extension analysis of the region encoding ORFs Bm42, Lef-10, and VP1054 revealed that transcription initiated independently for each of them. Transcription of *Bm*42 initiated from all the three canonical TAAG late transcription motifs located immediately upstream of the ORF. Although the upstream sequences of *lef*-10 as well as *vp*1054 lacked the known consensus early (CAGT)- or late (TAAG)-transcription start motifs, a TATA box-like sequence (TATAAAA) was present 20 nt upstream of the *lef*-10 transcription start site, identified here as "CGCG" motif present 163 nt upstream of the +1 ATG of the gene. This CGCG start-site motif, in the BmNPV-BGL strain, a local isolate of BmNPV used here (Palhan and Gopinathan, 1996), however, differed slightly from the BmNPV T3 strain (GenBank Accession No. L33180), which corresponded to a tetranucleotide stretch of C residues (CCCC). The primer extension results showed that all the transcripts corresponding to the individual ORFs encoded polycistronic messages. This inference was also confirmed by Northern analysis using the *Bm*42-specific probe. RT-PCR analysis revealed that while *Bm*42 transcript was a late one, the transcripts for the other two ORFs were detected early. This was also predicted based on the primer extension results because *Bm*42 5′ ends were mapped to three distinct TAAG motifs, and TATA box-like sequences were present upstream to the *lef*-10 start site. However, since the *lef*-10 transcript reads through the downstream *vp*1054 gene, signals in the *vp*1054 region are likely to be detected early in infection even though it might actually be a late gene, the encoded product being identified as a viral structural component in AcMNPV (Olszewski and Miller,

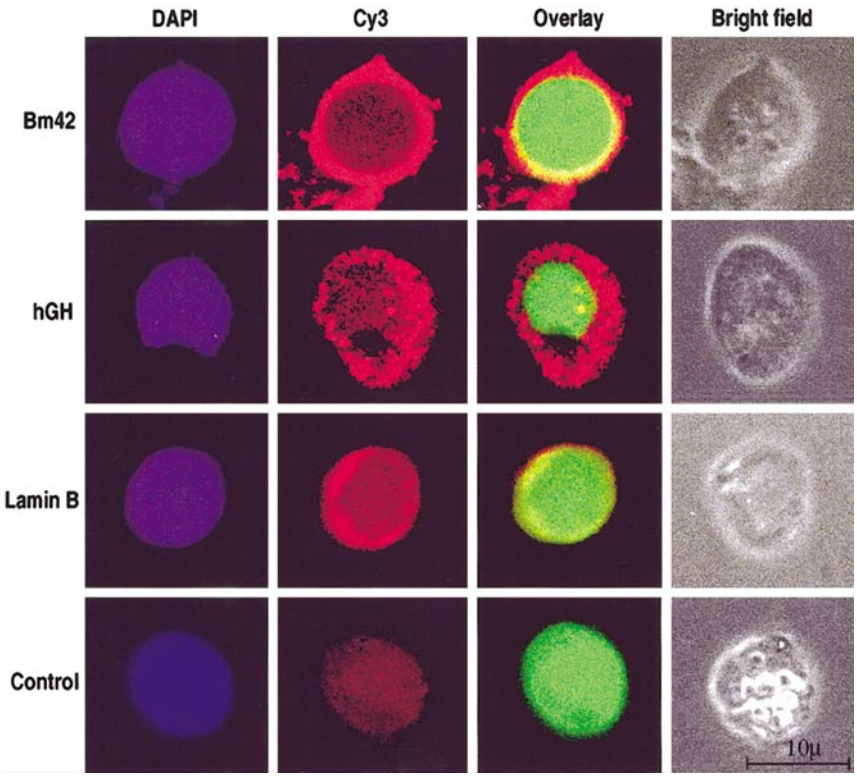


FIG. 5. Immunolocalization of Bm42 in BmNPV-infected BmN cells. BmN cells uninfected or infected with BmNPV were harvested 36 h.p.i. and immunostained for Bm42 using rabbit polyclonal antisera raised against the bacterially overexpressed and purified protein in rabbit. Staining for cytosolic marker (human growth hormone, hGH, expressed on infection of cells by a recombinant BmNPV overexpressing hGH under the polyhedrin promoter, Sumathy *et al.,* 1996) and a nuclear inner membrane marker (mouse Lamin B) were used as controls. A negative control, lacking treatment with anti-Bm42 antiserum, was also performed. The antibody reaction was detected using secondary antibody, goat anti-rabbit IgG-Cy3 conjugate, in all cases. Nuclei were counterstained using the DNA-specific fluorescent dye, DAPI. The overlays of the antibody and nuclear stainings, and bright field views of the same, are also shown.

1997). There were no TATA-like sequences in the immediate upstream region of *vp*1054 transcription start site, but it was generally AT-rich.

The RACE reaction performed to map the 3′ ends of the multiple transcripts in this region identified that termination occurred only at the distal-most polyadenylation site (marked A3), and the other two proximal polyadenylation sequences were not utilized. The mapping of the 3′ end also supported the 2.2-kb size of the *Bm*42 transcript, estimated by Northern blotting. The canonical globin polyadenylation signal sequence is effectively recognized in baculoviruses and most of the late and very late transcripts also harbor the AAUAAA motif near their 3′ ends (Westwood *et al.,* 1993). It was, therefore, believed that polyadenylation of the late transcripts and cleavage is brought about by the conventional mechanism operating in the host. In eukaryotes the polyadenylation machinery is targeted to the nascent transcripts by interactions with the carboxyl-terminal domain (CTD) of RNA polymerase II (Dantonel *et al.,* 1997; Hirose and Manley, 1998).

Recent studies in AcMNPV (Jin and Guarino, 2000) have shown that in baculoviruses the 3′-end processing activity is an inherent property of the RNA polymerase complex itself. However, none of the identified associated subunits of baculoviral RNA polymerase, viz., LEF-4, LEF-8, LEF-9, and P47 (Guarino *et al.,* 1998), that bring about initiation from late and very late gene promoters, harbor CTD-like domains. The *in vitro* assays with purified baculovirus RNA polymerase revealed that the 3′ ends were formed by termination after transcription of a T-rich region rather than by the cleavage mechanism. T-rich sequence was essential for termination and polyadenylation and the polyadenylation signal sequence (AATAAA) as well as the GT-rich region (essential for polyadenylation in most eukaryotes) were not essential (Jin and Guarino, 2000). The presence of the polyadenylation signal, AATAAA sequence in the AT-rich region near the termination point, was considered a mere coincidence.

Our results on 3′-end mapping revealed that of the three putative polyadenylation signal sequences identified in the Bm42 region, only the distal-most signal, harboring a T-rich stretch immediately downstream, was utilized as the termination site. In fact only A3 harbored the GT-rich region immediately downstream of the AAUAAA sequence. Therefore, the A3 site was capable of serving as the polyadenylation site at early time points

when the host polymerase was functioning, as well as at later times when polyadenylation is mediated by the virally encoded polymerase.

Multiple transcription initiation points and a single termination point were identified for the various transcripts from this region. Moreover, the results from RT-PCRs on the RNA synthesized in the presence and absence of the DNA replication inhibitor suggested the presence of early and late transcripts where the shorter transcripts (for *lef*-10 and *vp*1054) were expressed prior to the longer ones (for *Bm*42). Taken together, the results that transcription initiated at multiple points for the various ORFs in the *Bm*42 gene cluster in a temporal fashion and only a single transcription termination site was detected, we propose that this region of BmNPV exhibits the phenomenon of promoter occlusion. In this situation, the host transcription machinery initiating from early promoter motifs (TATA or AT-rich sequences in the vicinity of transcription start site) are prevented access to the transcription-initiation site by the virally encoded polymerase initiating at the late transcription start motif(s) TAAG, located farther upstream.

The *Bm*42-encoded protein was primarily localized to the cytoplasm of the infected cell. Bm42 also appeared to be a structural component of the budded virion because the protein was detected by Western blots in the purified virus preparations. However the protein was not detected in polyhedra-derived virions or with the polyhedral matrix. Despite the fact that the virus assembly takes place in the nucleus, the presence of Bm42 predominantly in the cytoplasm and closer to the outer nuclear membrane suggested that the budding virions should be picking up the protein during their exit from nucleus through the cytoplasm. Cytoplasmic localization of the polyhedral enveloped-associated 32-kDa protein in *Orgyia pseudotsugata* MNPV (multinucleocapsid nucleopolyhedrovirus)-infected cells, and the location of the virally encoded 16-kDa protein which does not associate with the budded virion or the polyhedral matrix, close to cytoplasm-nuclear membrane, have been reported (Gombart *et al.,* 1989; Gross *et al.,* 1993). The physical properties of the bacterially expressed protein such as its extreme tendency to aggregate under a variety of conditions, as well as the presence of abundant β-sheeted structures as revealed from the CD structural analysis of the purified protein (data not presented), were in conformity to the notion that Bm42 is a structural component of the virus. There were no recognizable membrane localization sequences or other known motifs present in Bm42 except for the presence of a potential ring zinc finger domain. Such motifs are implicated in protein multimerization. Bm42 is evolutionarily conserved in all the known baculoviruses sequenced so far, with the exception of *Culex nigripalpus* NPV (Moser *et al.,* 2001; Afonso *et al.,* 2001). Cuni NPV, however, is an unusual member of the *Baculoviridae* family which exhibits similarities in some aspects such as the rate of amino acid changes in highly conserved genes, to Nudiviruses, a group that has been excluded from *Baculoviridae* (Afonso *et al.,* 2001).

In earlier efforts, Gomi *et al.* (1999) could not generate BmNPV mutants having disruption in *lef*-10. In that study, however, *Bm*42 was mistakenly identified as *lef*-10 and the disruption using the *hsp*-β-gal cassette engineered at the *Bsm*I site was located in the C-terminus of ORF Bm42. Although the site of disruption was in the C-terminal-coding region of *Bm*42, it was still within 5′-UTR of *lef*-10, and therefore, possibly affected the expression of both *Bm*42 and *lef*-10. *Bm*42 appears to be an essential gene for virus production.

MATERIALS AND METHODS

Cell line and virus

B. mori-derived cell line, BmN, was propagated at 27°C in TC-100 medium supplemented with 10% fetal bovine serum (GIBCO-BRL). For all temporal expression profile as well as transient transfection studies, the BmNPV-BGL strain (a local isolate of BmNPV, Palhan and Gopinathan, 1996) was used. The virus stocks were maintained and titered according to the standard protocols (O'Reilly *et al.,* 1992). Budded virions were purified from culture supernatant of BmN cells (10^8) infected with BmNPV (m.o.i. 10) at 72 h.p.i. The culture supernatant was centrifuged at 10,000 rpm for 10 min to remove the cells and the budded virions in the supernatant were harvested by ultracentrifugation on a 25% sucrose cushion at 25,000 rpm for 1 h. Polyhedra-derived viruses (occluded virus) and the polyhedral matrix of BmNPV were isolated essentially as described for OpMNPV (Gombart *et al.,* 1989; Gross *et al.,* 1993). Briefly, 1 mg of purified BmNPV polyhedral bodies (isolated from BmNPV infected *B. mori* larvae) were heated at 70°C for 20 min to inactivate the proteases and disrupted in dilute alkaline solution (50 mM Na_2CO_3 and 50 mM NaCl) at 60°C for 5 min. The supernatant after centrifugation at 12,000 *g* for 10 min at 4°C was taken as the source of occluded viruses and solubilized matrix. The pellet was treated with 0.5% SDS in the presence or absence of 1% β-mercaptoethanol. All the supernatant and pellet fractions were analyzed by Western blots for the presence of Bm42.

Generation of plasmid constructs

The synthetic oligonucleotides used as primers for PCR amplification, cloning, sequencing, and primer extensions are listed in Table 1. The plasmid clones pBmXL and pBmXN harboring 2.2- and 1.32-kb genomic fragments L and N, respectively, were obtained from an *Xho*I library of BmNPV-BGL. For expression in bacteria as a His-tagged, thioredoxin fusion, ORF Bm42 was PCR amplified from BmNPV genomic DNA as a 417-bp *Bam*HI-

*Sal*I fragment (using primers P1 and P4) and cloned into *Bam*HI-*Xho*I sites in the plasmid vector pET32a. The construct pBm42C utilized for *lef*-10 tss mapping by RNase protection was generated by an *Xho*I deletion and self-ligation of the full-length clone of *Bm*42 in pBS SK+ (inserted at the *Eco*RV site). The full-length ORF Bm42 lacking the thioredoxin fusion was expressed as a *Nde*I-*Sal*I fragment in pET21a. Bm42 was also expressed without His-tag and thioredoxin fusion in bacteria and the proteins predominantly present in the inclusion bodies were partially solubilized and renatured by treatment with guanidine hydrochloride.

RNase protection, primer extension, Northern blotting, and 3′-RACE

Total RNA from uninfected- and BmNPV-infected BmN cells at various times postinfection was isolated by guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987) and treated with DNase I (RNase free; 10 U/10^7 cells). The expression profile of *Bm*42 was analyzed by RNase protection assays using the appropriate complementary RNA probes. Antisense riboprobes of high specific activity were generated *in vitro* from the cloned BmNPV genomic DNA fragments in plasmid pBSSK+ using T3 or T7 RNA polymerase as appropriate, in the presence of radiolabeled [α-^{32}P]UTP. Total RNA (20–40 μg) was coprecipitated with the corresponding antisense riboprobe (1.5 $\times$ 10^5 cpm) in the presence of 200 mM NaCl and 20 μg of carrier DNA, using 2.5 vol ethanol. Following hybridization at 50°C overnight in the presence of 50% formamide, the RNase digestion mix containing RNase A (2U) and RNase T1 (1U) was added and after 1 h at 37°C the samples were precipitated in the presence of 10 μg yeast tRNA (added as carrier) and 2.5 vol ethanol. The RNase-protected samples were analyzed by electrophoresis on 7 M urea–6% acrylamide gels and visualized by autoradiography.

Transcription start sites were mapped by primer extension analysis. Total RNA (20–40 μg) was annealed to 5 pmol of the appropriate primer and reverse transcription was performed using Superscript II Reverse transcriptase (GIBCO-BRL) in the presence of [α-^{32}P]dATP (10 μCi; specific activity, 3000 Ci/mmol) and 100 pmol each of dCTP, dGTP, and dTTP for 5 min at 42°C. This was followed by extension reactions for 5 min in the presence of vast excess of all four dNTPs (200 μM each) and the reactions were terminated using 80% formamide gel-loading dye containing 200 μM EDTA. The primer-extended products were analyzed on 7 M urea–6% acrylamide gels together with appropriate DNA sequencing ladders for sizing and detected by autoradiography.

Northern blotting was carried out as described in Sambrook *et al.* (1989). Total RNA (40 μg) isolated from control as well as BmNPV-infected BmN cells was separated on a 1.2% MOPS formaldehyde agarose gel and transferred to N^+ nylon membrane (Amersham Pharmacia Biotech). The blot was probed using radiolabeled N-terminal *Bm*42-specific probe, generated by random priming. Following hybridization and cross-linking, the blots were washed at a final stringency of 0.1$\times$ SSC and 0.1% SDS and autoradiographed.

The 3′-end mapping was carried out by RACE. Reverse transcriptions were performed with total RNA using 3′-RACE adapter primer, 5′GGCCACGCGTCGACTAGTAC $(T)_{17}$ 3′ and Superscript II Reverse transcriptase at 42°C for 1 h. Following RNase H treatment at 30°C for 30 min, PCR amplification was carried out with 1/10 volume of the above reaction and *Vent* DNA polymerase using P1 (encompassing the +1 ATG of ORF Bm42) and the 3′-RACE anchor primer 5′ GGCCACGCGTCGACTAGTAC 3′. This was followed by two rounds of amplification with nested primers, first with P7 and then with P8 (located in the ORF Bm44 coding region) and 3′-RACE anchor primer. The amplified product was cloned in pGEMT-Easy (Promega) and sequenced using T7 primer, to precisely map the transcription termination site.

Raising polyclonal antisera and Western blotting

Polyclonal antiserum was raised in rabbit against ORF Bm42 expressed as a thioredoxin fusion protein with a C-terminal His-tag (clone pTrxABm42) in *E. coli*, strain BL-21. The bacteria were grown at 22°C and induced with 25 μM IPTG at log phase (OD_{600nm} of 0.6). After 8 h, the cells were harvested and the soluble fraction of proteins was bound to a Ni-NTA agarose column at 4°C. The column was washed with 50 mM imidazole and the bound proteins were eluted with 500 mM imidazole. The purified protein (800 μg) was injected into a rabbit in the presence of Freund's complete adjuvant followed by three rounds of boosters, each with 500 μg of the purified protein (in Freund's incomplete adjuvant) administered at an interval of 10 days. The rabbit serum was tested for the presence of antibodies and stored at 4°C in the presence of 0.2% sodium azide. For analyzing the temporal synthesis of Bm42, uninfected as well as BmNPV-infected BmN cells (1 $\times$ 10^5) were suspended in SDS gel loading buffer [50 mM Tris (pH 6.8), 2% SDS, 1% β-ME, and 10% glycerol] and analyzed on a 0.1% SDS–8% polyacrylamide gel. Following electrophoresis, the proteins were electrophoretically transferred on to PVDF membrane at 1.0 mA/cm^2 for 1 h and the membrane was blocked with 3% gelatin overnight. The blot was probed with 1:1000 dilution of the rabbit anti-Bm42 antiserum followed by washing and incubation with secondary anti-rabbit goat antibody, conjugated to HRP. After extensive washing, the blot was developed using ECL+Plus kit (Amersham Pharmacia Biotech).

Immunofluoresence microscopy

Subcellular localization of viral proteins was determined by immunofluorescence microscopy. BmN cells (2×10^6) were harvested at the time point of peak expression (36 h for Bm42 and 48 h for *polh* based expression of human growth hormone) and washed with 1× PBS. Cells were fixed in 4% paraformaldehyde and 0.5% DMSO on ice for 30 min and then washed three times with 1× PBS. Permeabilization was carried out in the presence of 40 mM methylamine and 0.1% Triton X-100 for 30 min on ice. Following blocking with 2.5% NGS (normal goat serum) and 2% BSA overnight, primary antisera precleared with uninfected cell lysate was added and incubated for 8 h at 4°C. The samples were subsequently washed with 0.1% BSA and 0.1% Triton X-100 and blocked again with 1% BSA before incubating with goat anti-rabbit IgG-Cy3 conjugate (1:1000 dilution) for 8 h at 4°C. The cells were washed twice with 1× PBS containing 0.1% Triton X-100 and stained with DAPI (DNA specific fluorescent dye) for 10 min at 4°C and then washed extensively with the same buffer as before. Samples were mounted in 50% glycerol in PBS and observed under confocal fluorescence microscopy.

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REFERENCES

Acharya, A., and Gopinathan, K. P. (2001). Identification of an enhancer-like element in the polyhedrin gene upstream region of *Bombyx mori* nucleopolyhedrovirus. *J. Gen. Virol.* **82,** 2811–2819.

Afonso, C. L., Tulman, E. R., Lu, Z., Balinsky, C. A., Moser, B. A., Becnel, J. J., Roct, D. L., and Kutish, G. F. (2001). Genome sequence of a baculovirus pathogenic for *Culex nigripalpus. J. Virol.* **75,** 11157–11165.

Ayres, M. D., Howard, S. C., Kuzio, J., Lopez-Ferber, M., and Possee, R. D. (1994). The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. *Virology* **202,** 586–605.

Bateman, E., and Paule, M. R. (1988). Promoter occlusion during ribosomal RNA transcription. *Cell* **54,** 985–992.

Blissard, G. W., and Rohrmann, G. F. (1990). Baculovirus diversity and molecular biology. *Ann. Rev. Entomol.* **35,** 127–155.

Carthew, R. W. (2001). Gene silencing by double-stranded RNA. *Curr. Opin. Cell Biol.* **13,** 244–248.

Chomczynski, P., and Sacchi, N. (1987). Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156–159.

Dantonel, J., Murthy, K. G. K., Manley, J. L., and Tora, L. (1997). Transcription factor TFIID recruits factor CSPF for formation of 3′ end of mRNA. *Nature* **389,** 399–402.

Friesen, P. D., and Miller, L. K. (1985). Temporal regulation of baculoviral RNA: Overlapping early and late transcripts. *J. Virol.* **54,** 392–400.

Friesen, P. D., and Miller, L. K. (1986). The regulation of baculovirus gene expression. *Curr. Topics Microbiol. Immunol.* **131,** 31–49.

Fuchs, L. Y., Woods, M. S., and Weaver, R. F. (1983). Viral transcription during AcNPV infection: A novel RNA polymerase induced in *Spodopter frugiperda* cells. *J. Virol.* **48,** 641–646.

Gombart, A. M., Pearson, M. N., Rohrmann, G. F., and Beaudreau, G. S. (1989). A baculovirus polyhedral envelope-associated protein: Genetic location, nucleotide sequence and immunological characterization. *Virology* **169,** 182–193.

Gomi, S., Majima, K., and Maeda, S. (1999). Sequence analysis of the genome of *Bombyx mori* nucleopolyhedrovirus. *J. Gen. Virol.* **80,** 1323–1337.

Greger, I. H., Demarchi, F., Giacca, M., and Proudfoot, N. J. (1998). Transcriptional interference perturbs the binding of Sp1 to the HIV-1 promoter. *Nucleic Acids Res.* **26,** 1294–1301.

Gross, C. H., Wolgamot, G. M., Russell, R. L. Q., Pearson, M. N., and Rohrmann, G. F. (1993). A baculovirus encoded 16 kDa glycoprotein localizes near the nuclear membrane of infected cells. *Virology* **192,** 386–390.

Guarino, L. A., Xu, B., Jin, J., and Dong, W. (1998). A virus encoded RNA polymerase purified from baculovirus-infected cells. *J. Virol.* **72,** 7985–7991.

Hashimoto, Y., Hayakawa, T., Ueno, Y., Fujita, T., Sano, Y., and Matsumoto, T. (2000). Sequence analysis of the *Plutella xylostella* granulovirus genome. *Virology* **275,** 358–372.

Hausler, B., and Sommerville, R. L. (1979). Interaction *in vivo* between strong closely spaced constitutive promoters. *J. Mol. Biol.* **127,** 353–356.

Hayakawa, T., Rohrmann, G. F., and Hashimoto, Y. (2000). Patterns of genome organization and content in lepidopteran baculoviruses. *Virology* **278,** 1–12.

Hirose, Y., and Manley, J. L. (1998). RNA polymerase II is an essential polyadenylation factor. *Nature* **395,** 93–96.

Jin, J., and Guarino, L. A., (2000). 3′ end formation of baculovirus late RNAs. *J. Virol.* **74,** 8930–8937.

King, L. A., and Possee, R. D. (1992). "The Baculovirus Expression System—A Laboratory Guide." Chapman and Hall, London, UK.

Lu, A., and Miller, L. K. (1994). Identification of three late gene expression factors within the 33.8–43.4 map-unit region of *Autographa californica* nuclear polyhedrosis virus. *J. Virol.* **68,** 6710–6718.

Lubbert, H., and Doerfler, W. (1984a). Transcription of overlapping set of RNAs from the genome of *Autographa californica* nuclear polyhedrosis virus: A novel method for mapping RNAs. *J. Virol.* **52,** 255–265.

Lubbert, H., and Doerfler, W. (1984b). Mapping of early and late transcripts encoded by the AcNPV genome: Is viral RNA spliced? *J. Virol.* **50,** 497–506.

Maeda, S. (1989a). Expression of foreign genes in insects using baculovirus vectors. *Ann. Rev. Entomol.* **34,** 351–372.

Maeda, S. (1989b). Gene transfer vectors of a baculovirus, *Bombyx mori* nuclear polyhedrosis virus and their use for expression of foreign genes in insect cells. *In* "Invertebrate Cell System Applications" (J. Mitsuhashi, Ed.), pp. 167–182. CRC Press, Boca Raton, FL.

Miller, L. K. (1992). Baculoviruses as gene expression vectors. *Ann. Rev. Microbiol.* **42,** 177–199.

Moser, B. A., Becnel, J. J., White, S. E., Afonso, C. L., Kutish, G. F., Shankar, S., and Almira, E. (2001). Morphological and molecular evidence that *culex nigripalpus* baculovirus is an unusual member of the family *Baculoviridae. J. Gen. Virol.* **82,** 283–297.

Moss, T., Larose, A. M., Mitchelson, K., and Leblanc, B. (1992). Readthrough enhancement and promoter occlusion on the ribosomal genes of Xenopus laevis. *Biochem. Cell Biol.* **70,** 324–331.

Oellig, C., Happ, B., Muller, T., and Doerfler, W. (1987). Overlapping sets of viral RNAs reflect the array of polypeptides in the *Eco*RI J and N fragments (map positions 81.2 to 85.0) of the AcMNPV genome. *J. Virol.* **61,** 3048–3057.

Olszewski, J., and Miller, L. K. (1997). Identification and characterization

of a baculovirus structural protein, vp1054, required for nucleocapsid formation. *J. Virol.* **71,** 5040–5050.

O'Reilly, D. R., Miller, L. K., and Luckow, V. E. (1992). *Baculovirus Expression Vectors—A Laboratory Manual* (W. H. Freeman and Co., Eds.) New York.

Palhan, V. B., Sumathy, S., and Gopinathan K. P. (1995). Baculovirus mediated high level expression of luciferase in silkworm cells and larvae. *Biotechniques* **19,** 97–105.

Palhan, V. B., and Gopinathan, K. P. (1996). Characterization of a local isolate of *Bombyx mori* nuclear polyhedrosis virus. *Curr. Sci.* **70,** 147–153.

Possee, R. D. (1997). Baculoviruses as expression vectors. *Curr. Opin. Biotech.* **8,** 569–572.

Possee, R. D., and Howard, S. C. (1987). Analysis of the polyhedrin gene promoter of *Autographa californica* nuclear polyhedrosis virus. *Nucleic Acids Res.* **15,** 10233–10248.

Proudfoot, N. J. (1986). Transcriptional interference and termination between duplicated α-globin gene constructs suggests a novel mechanism for gene regulation. *Nature* **322,** 562–565.

Rankin, C., Ladin, B. F., and Weaver, R. F. (1986). Physical mapping of temporally regulated, overlapping transcripts in the region of the 10K protein gene in *Autographa californica* nuclear polyhedrosis virus. *J Virol.* **57,** 18–27.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sehgal, D., and Gopinathan, K. P. (1998). Recombinant *Bombyx mori* nucleopolyhedrovirus harboring green fluorescent protein. *Biotechniques* **25,** 997–1004.

Sumathy, S., Palhan, V. B., and Gopinathan, K. P. (1996). Expression of human growth hormone in silkworm larvae through recombinant *Bombyx mori* nuclear polyhedrosis virus. *Protein Exp. Purif.* **7,** 262–268.

Todd, J. W., Passarelli, A. L., and Miller, L. K. (1995). Eighteen baculovirus genes, including *lef11, p35, 39K* and *p47,* support late gene expression. *J. Virol.* **69,** 968–974.

Vales, L. D., and Darnell, J. E., Jr. (1989). Promoter occlusion prevents transcription of adenovirus polypeptide IX mRNA until after DNA replication. *Genes Dev.* **3,** 49–59.

Westwood, J. A., Jones, I. M., and Bishop, D. H. (1993). Analyses of alternative poly(A) signals for use in baculovirus expression vectors. *Virology* **195,** 90–99.

Yang, L., Stelter, D. A., and Wever, R. F. (1991). Structural comparison of the *Autographa californica* nuclear polyhedrosis virus-induced RNA polymerase and the three nuclear polymerases from the host, *Spodoptera frugiperda. Virus. Res.* **20,** 251–264.